APPENDIX

p38γ and p38δ reprogram liver metabolism by modulating neutrophil infiltration

Bárbara González-Terán[1#], Nuria Matesanz[1#], Ivana G. Nikolic[1#], María Angeles Verdugo[2, 1], Vinatha Sreeramkumar[1], Lourdes Hernández-Cosido[3], Alfonso Mora[1], Georgiana Crainiciuc[1], María Laura Sáiz[1], Edgar Bernardo[1], Luis Leiva-Vega[1], Elena Rodríguez[1], Victor Bondía[1], Jorge L. Torres[4], Sonia Perez-Sieira[5, 6], Luis Ortega[3], Ana Cuenda[2], Francisco Sanchez-Madrid[1], Rubén Nogueiras[5, 6], Andrés Hidalgo[1], Miguel Marcos[4], Guadalupe Sabio[1*].

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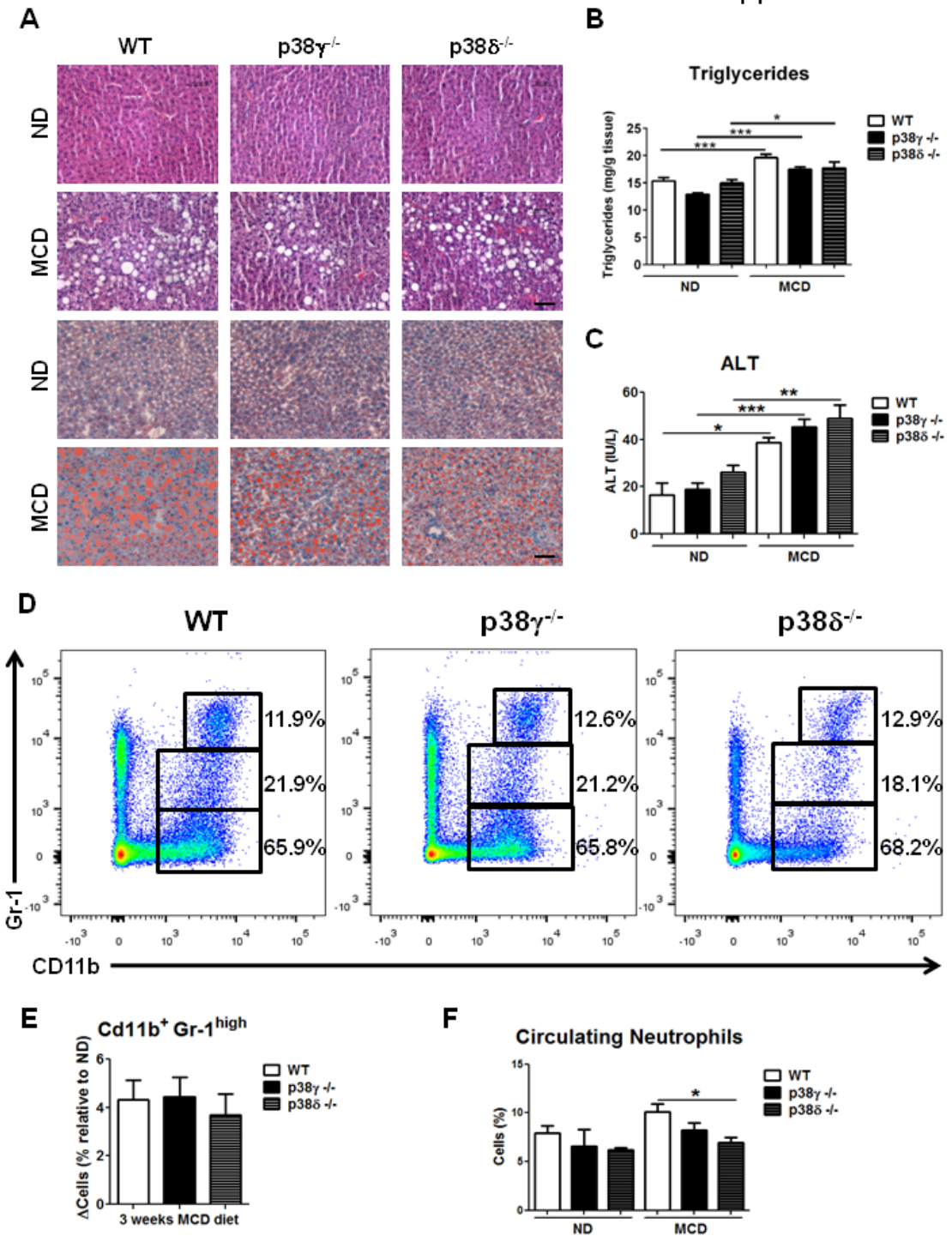
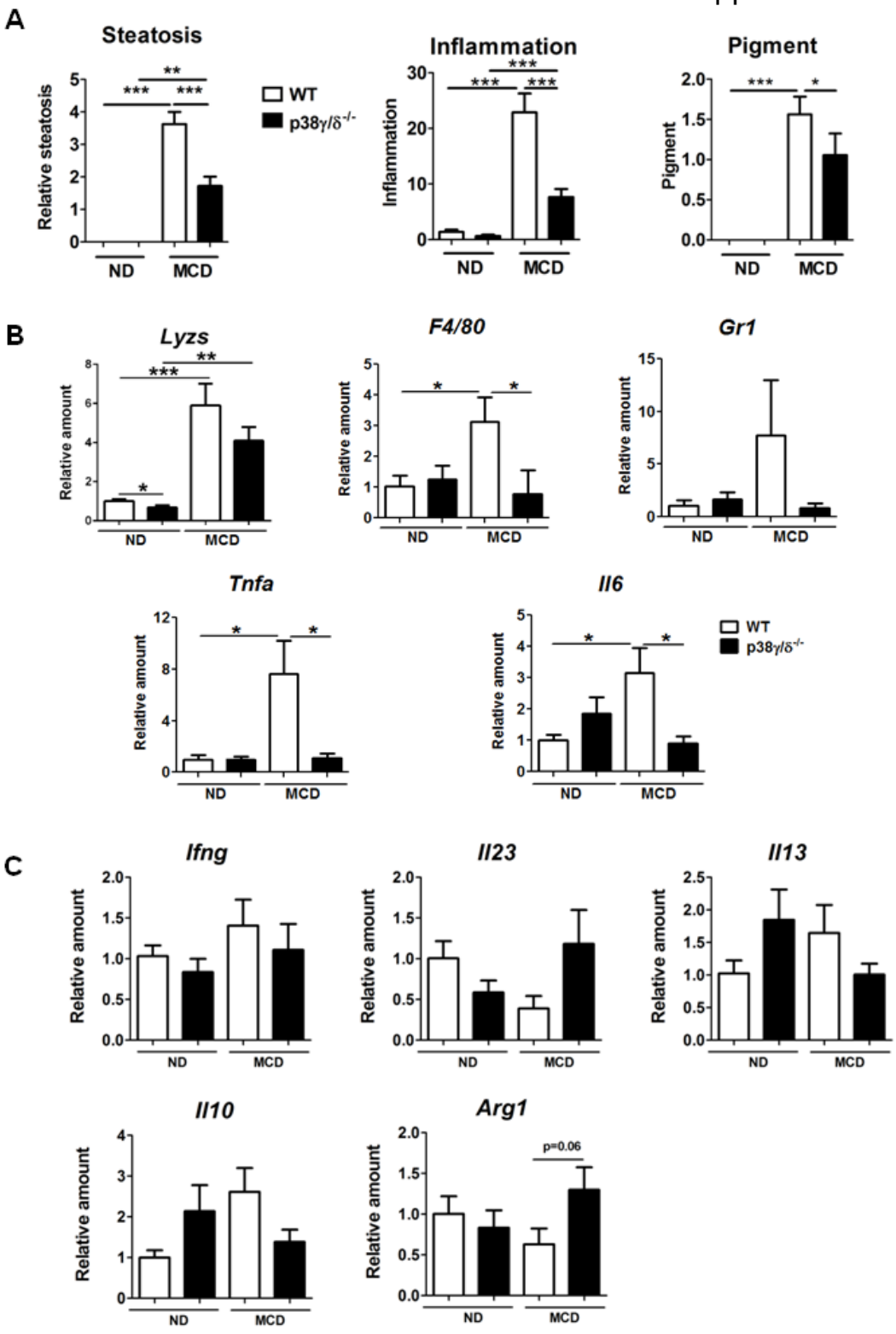
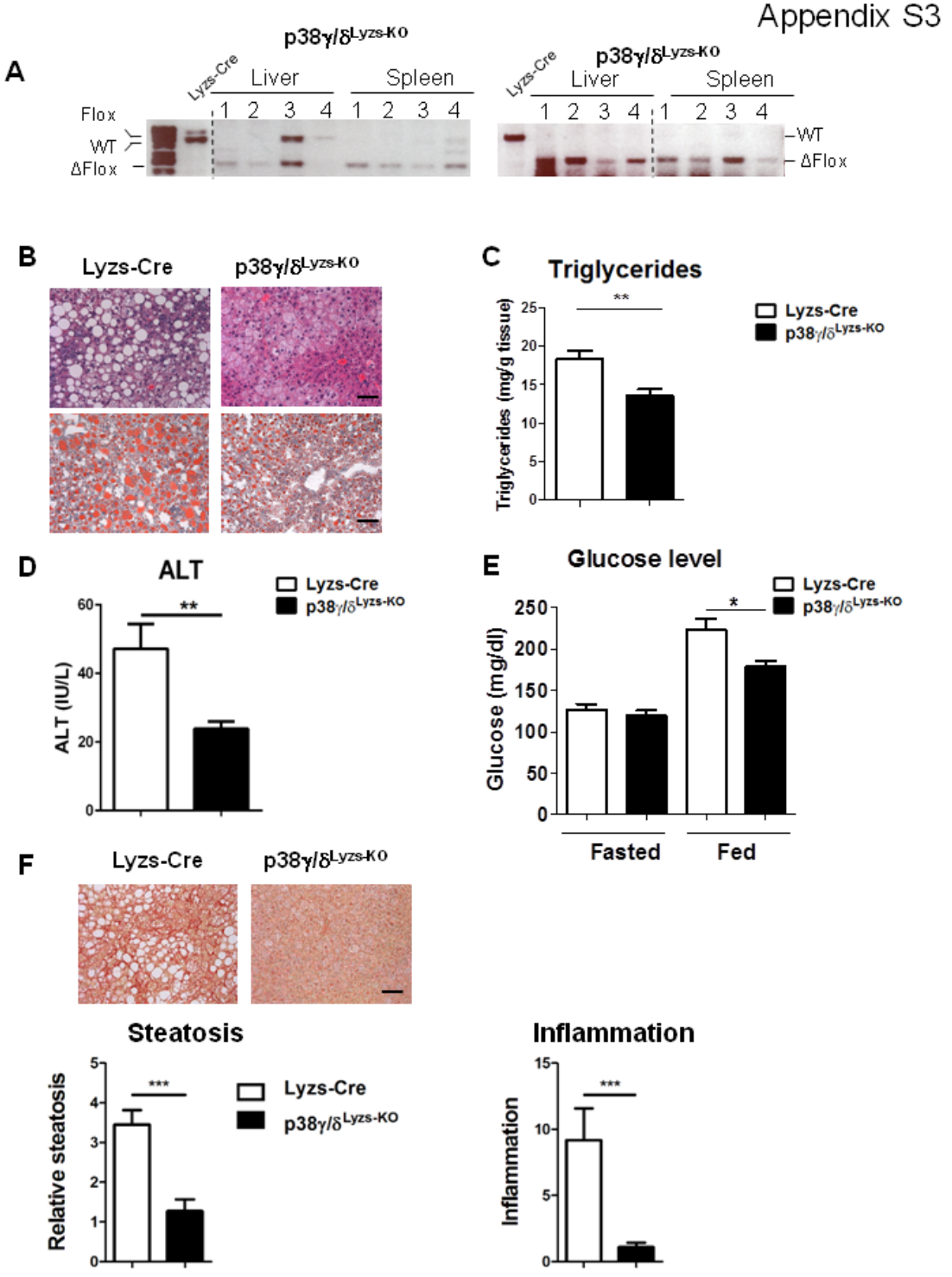


Appendix Fig. S1. p38γ$^{-/-}$ and p38δ$^{-/-}$ mice are partially protected against steatohepatitis.

WT, p38$\gamma^{-/-}$ and p38$\delta^{-/-}$ mice were fed a control diet (ND) or a diet deficient in methionine and choline (MCD) for 3 weeks. (**A**) Representative H&E and oil red stained liver sections. Scale Bar: 50μm. (**B**) Liver triglyceride and (**C**) plasma transaminase activity (ALT) at the end of the diet period. (**D-E**) Flow cytometry analysis of liver myeloid subsets (CD11b^{+} Gr-1^{high}, CD11b^{+} Gr-1^{intermediate}, CD11b^{+} Gr-1^{-}) isolated from WT, p38$\gamma^{-/-}$ and p38$\delta^{-/-}$ mice fed the MCD diet for 3 weeks. Representative dot plots are shown (**D**), and bar charts (**E**) show the diet-induced increase in each population relative to controls fed a ND as a percentage of the total intra-hepatic CD11b^{+} leukocyte population. (**F**) Neutrophils as a percentage of circulating leukocytes, measured in total blood in animals fed a MCD diet for 3 weeks. Data are means ± SEM. (n=5-10). *P<0.05; **P< 0.01; ***P< 0.001 (1-way ANOVA coupled to Bonferroni's post tests).

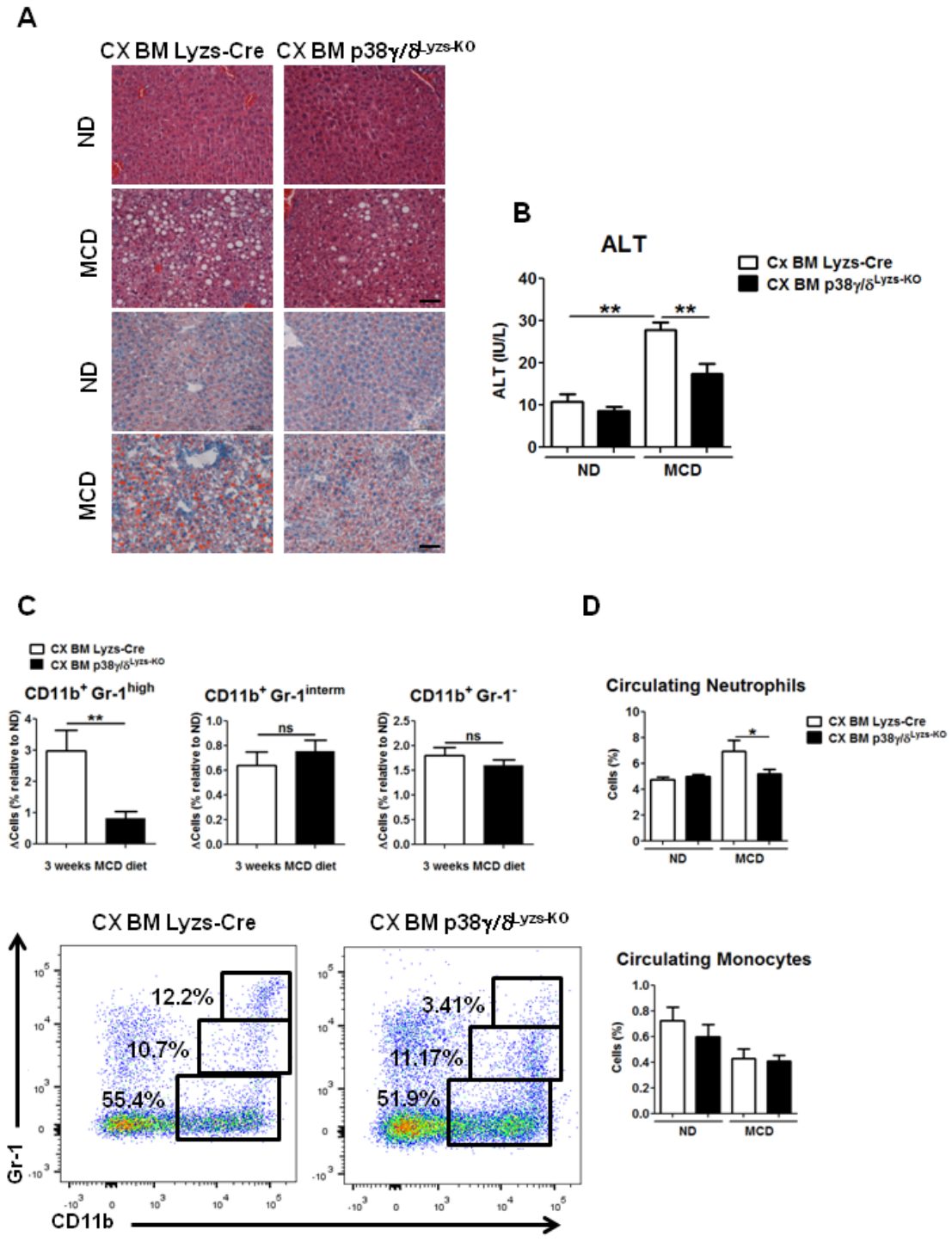

A
Steatosis
Relative steatosis
WT
p38γ/δ-/-
ND
MCD
Inflammation
Inflammation
Pigment
Pigment
B
Lyzs
F4/80
Gr1
Tnfa
Il6
Relative amount
C
Ifng
Il23
Il13
Il10
Arg1
p=0.06

Appendix Fig. S2. p38γ/δ$^{-/-}$ mice are protected against liver inflammation induced by MCD diet. WT, p38γ/δ$^{-/-}$ mice were fed a ND or the MCD diet for 3 weeks. (**A**) Quantification of liver H&E stained sections from WT and p38γ/δ$^{-/-}$ mice for steatosis, inflammation and pigment. Total RNA was extracted from livers, and (**B**) myeloid cell markers and cytokine mRNA levels and (**C**) M1 and M2 macrophages markers determined by qRT-PCR; mRNA expression was normalized to the amount of *Gapdh* mRNA. Data are means ± SEM. (n=5-10). *P<0.05; **P<0.01; ***P<0.001 (1-way ANOVA coupled to Bonferroni's post tests).



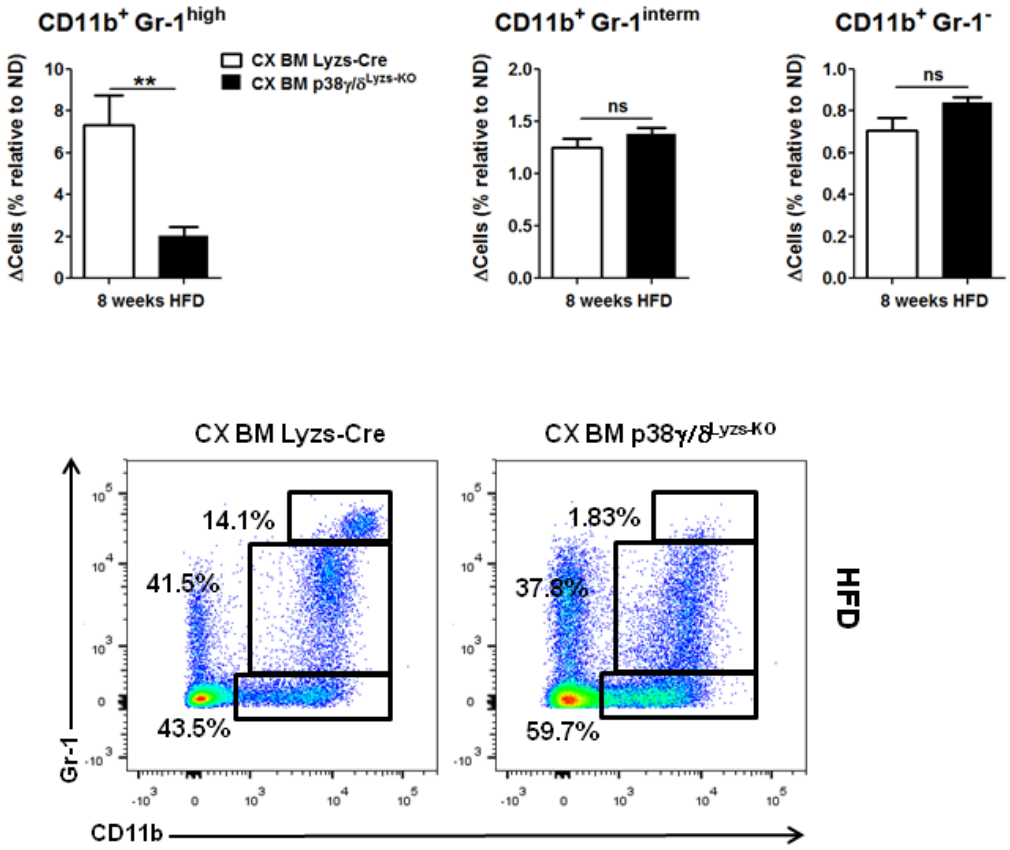
Appendix Fig. S3. p38γ/δ$^{Lyzs\text{-}KO}$ mice are protected against steatohepatitis induced by HFF. (A) PCR analysis of p38δ (left) and p38γ (right) deletion in macrophages (1), neutrophils (2), and dentritic cells (3, 4) infiltrated in liver and spleen from p38γ/δ$^{Lyzs\text{-}KO}$. **(B-F)** Lyzs-Cre and p38γ/δ$^{Lyzs\text{-}KO}$ mice were fed a high cholesterol, high saturated

fat, and high fructose (HFF) diet for 10 weeks. (**B**) Representative H&E and oil red stained liver sections (Scale Bar: 50μm) and (**C**) liver triglycerides (n=5-10). (**D**) Plasma ALT at the end of the diet period. (**E**) Basal blood glucose in overnight-fasted and fed Lyzs-Cre and p38γ/$\delta^{Lyzs\text{-}KO}$ mice (n=5-10). (**F**) Representative picrosirius red stained liver sections (Scale Bar: 50μm) and quantification of H&E stained liver sections for steatosis and inflammation. Data are means ± SEM. (n=5-10) *P<0.05; **P<0.01; ***P<0.001 refers to p38γ/$\delta^{Lyzs\text{-}KO}$ versus Lyzs-Cre; ##P<0.01; ###P<0.001 refers to ND versus HFD (1-way ANOVA coupled to Bonferroni's post tests or *t-test*).



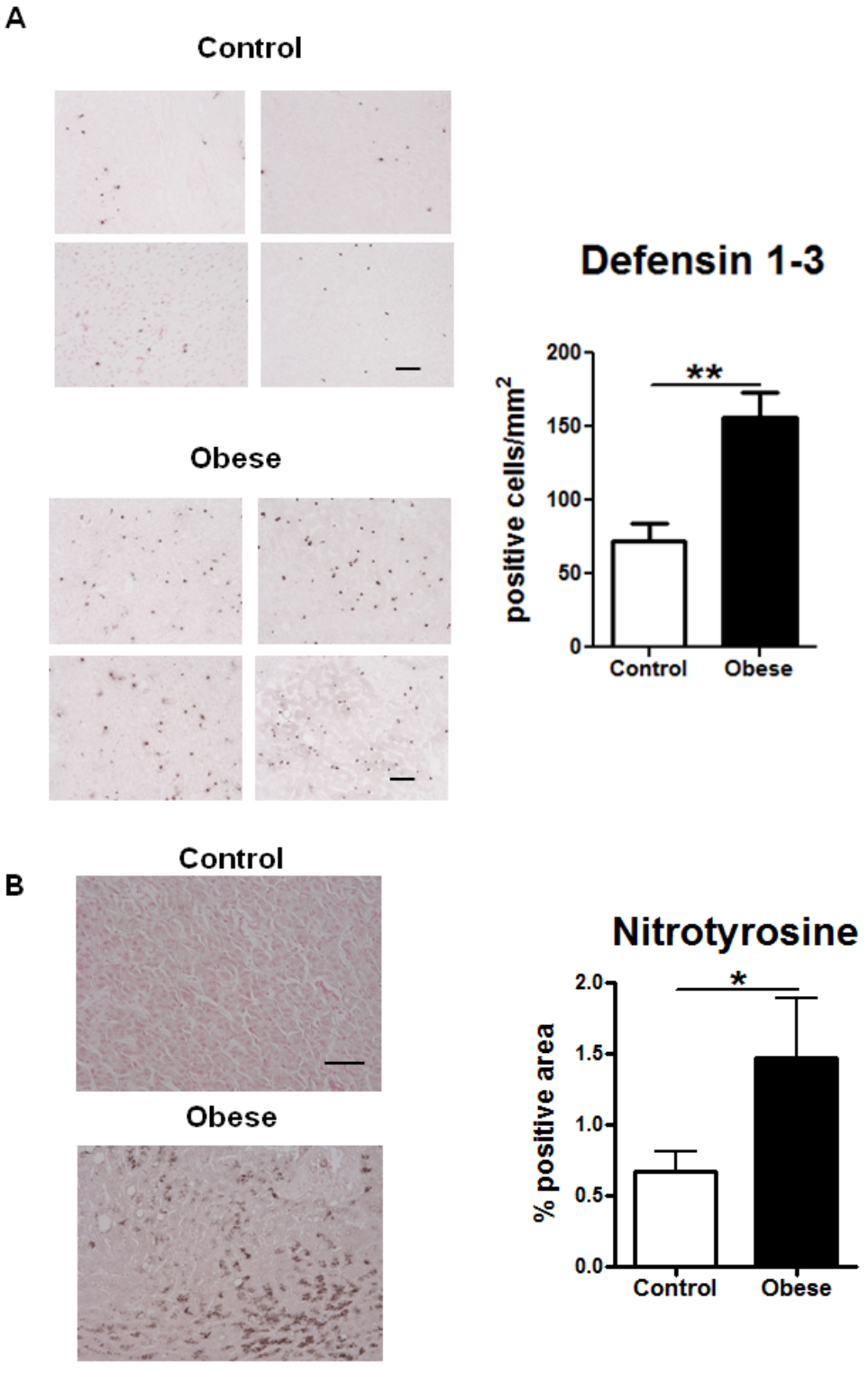
Appendix Fig. S4. Lyzs-Cre hematopoietic cells protect mice against MCD-diet–induced steatosis. Lethally irradiated WT mice were reconstituted with BM from Lyzs-

Cre (Cx BM Lyzs-Cre) or p38γ/$\delta^{Lyzs\text{-}KO}$ mice (Cx BM p38γ/$\delta^{Lyzs\text{-}KO}$). Two months after the transplant, mice were fed the MCD diet for 3 weeks. (**A**) Representative H&E and oil red-stained liver sections. Scale bar: 50 μm. (**B**) Serum transaminase ALT activity after MCD diet. (**C**) Flow cytometry analysis of liver myeloid subsets ($CD11b^+$ $Gr\text{-}1^{high}$, $CD11b^+$ $Gr\text{-}1^{intermediate}$, and $CD11b^+$ $Gr\text{-}1^-$) isolated from Cx BM Lyzs-Cre and Cx BM p38γ/$\delta^{Lyzs\text{-}KO}$ mice fed the MCD diet for 3 weeks. Representative dot plots are shown, and bar charts show the diet-induced increase in each population relative to controls fed a ND as a percentage of the total intra-hepatic $CD11b^+$ leukocyte population. (**D**) Neutrophils and monocytes as a percentage of circulating leukocytes, measured in total blood in animals fed a MCD diet for 3 weeks. Data are means ± SEM. (n=5-10) *P<0.05; **P<0.01 (1-way ANOVA coupled to Bonferroni's post tests or *t-test*).



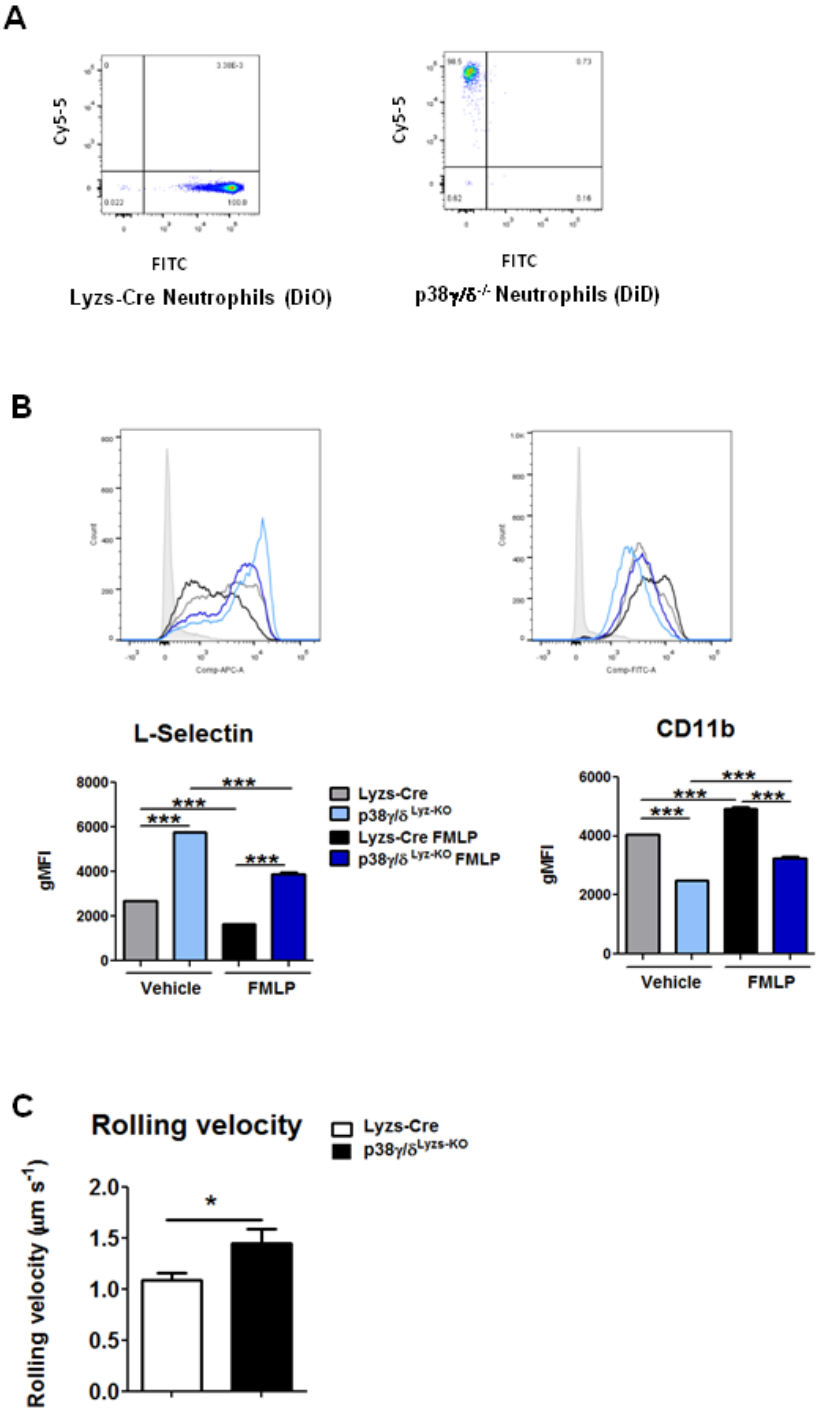
Appendix Fig. S5. Lyzs-Cre hematopoietic cells protect mice against HFD-induced liver neutrophil infiltration. Lethally irradiated WT mice were reconstituted with BM from Lyzs-Cre (Cx BM Lyzs-Cre) or p38γ/$\delta^{Lyzs\text{-}KO}$ mice (Cx BM p38γ/$\delta^{Lyzs\text{-}KO}$). Two

months after the transplant, mice were fed the HFD for 10 weeks. Flow cytometry analysis of liver myeloid subsets ($CD11b^+$ $Gr\text{-}1^{high}$, $CD11b^+$ $Gr\text{-}1^{intermediate}$, and $CD11b^+$ $Gr\text{-}1^-$) isolated from Cx BM Lyzs-Cre and Cx BM $p38\gamma/\delta^{Lyzs\text{-}KO}$ mice fed the HFD for 10 weeks. Representative dot plots are shown, and the bar charts show the diet-induced increase in each population relative to controls fed a ND as a percentage of the total intra-hepatic $CD11b^+$ leukocyte population. Data are means ± SEM. (n=5-10) **P<0.01 (*t-test*).



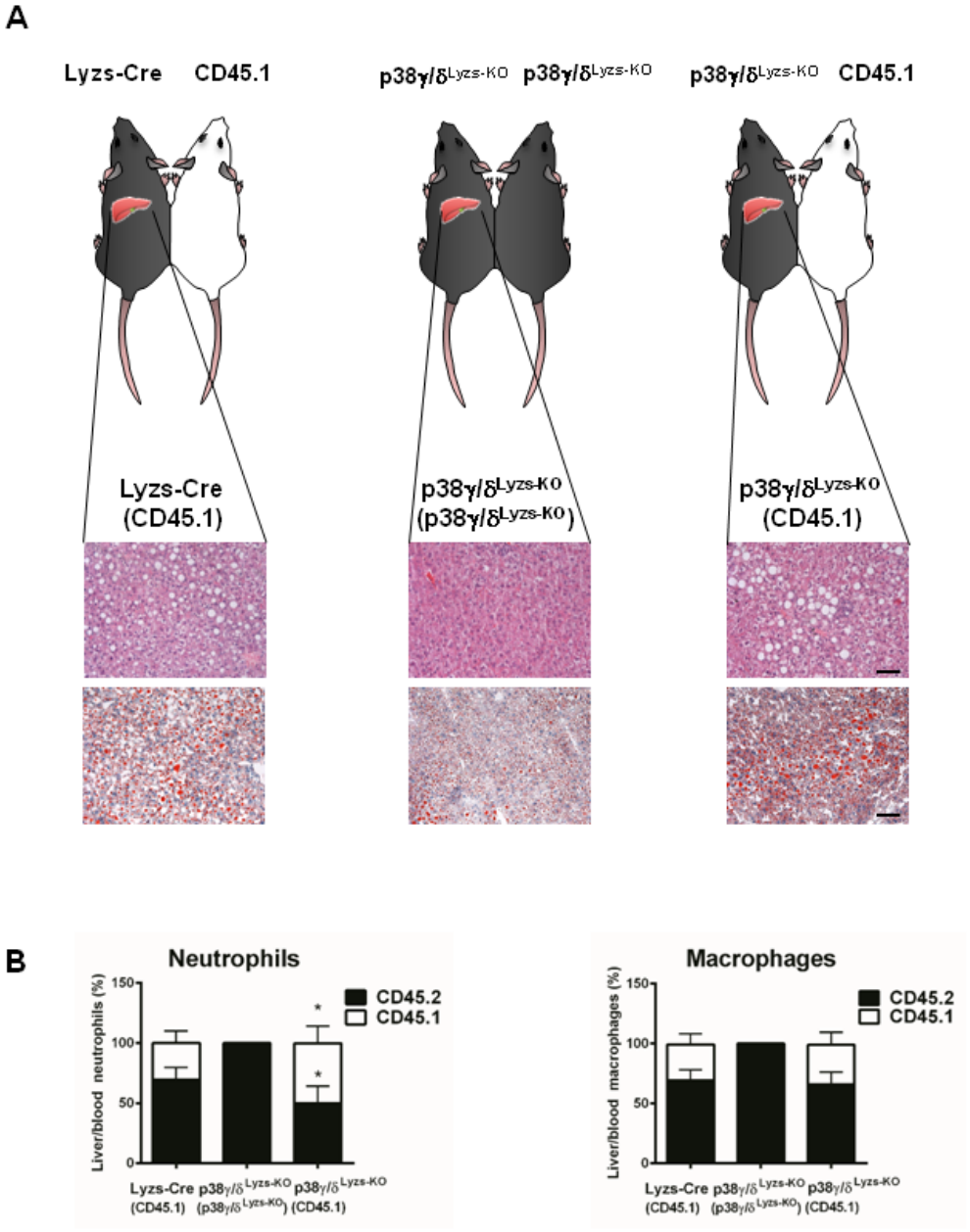
Appendix Fig. S6. Neutrophil infiltration during steatosis development. Liver biopsies from obese patients with NAFLD and from control patient without NAFLD

were stained (**A**) anti-human neutrophil defensin 1-3 (scale bar: 100 μm) and quantification of neutrophil infiltration by mm^2 of liver parenchyma. (**B**) Representative slides of liver biopsies stained with anti-nitrotyrosine antibody and quantification of neutrophil infiltration by mm^2 of liver parenchyma. Scale bar: 50 μm. Data are means ± SEM. (n=5-10) *P<0.05, **P<0.01 (*t-test*).



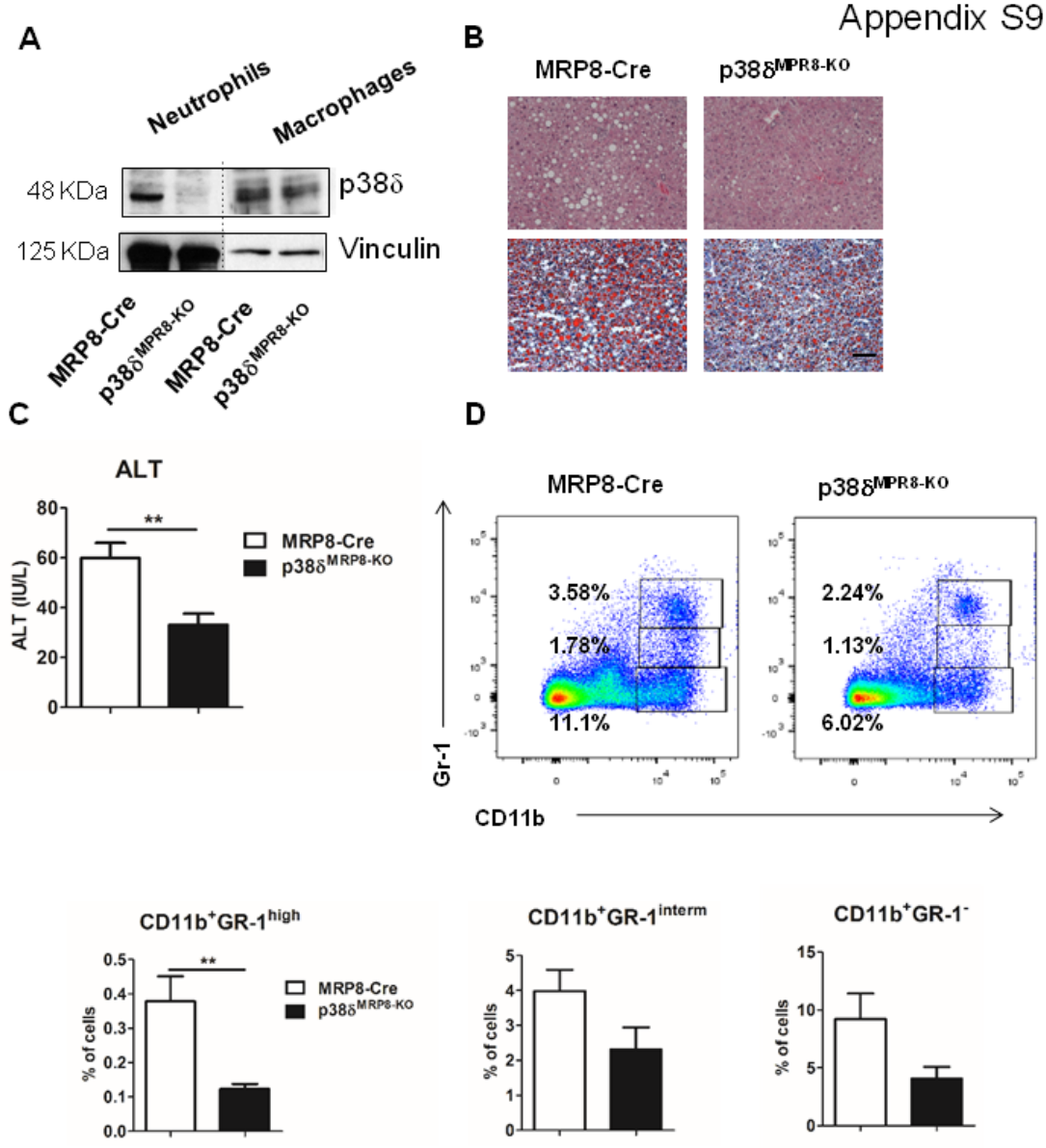
Appendix Fig. S7. Myeloid p38γ/δ deletion affects expression of L-Selectin and CD11b. (**A**) BM-isolated neutrophils isolated from Lysz-Cre mice and p38γ/$\delta^{Lyz\text{-}KO}$ transgenic mice labeled with DiO (Lyzs-Cre neutrophils) and DiD (p38γ/$\delta^{Lyzs\text{-}KO}$ neutrophils) were stained with DAPI and their survival were analyzed by flow

cytometry. Representative dot plots of DAPI-negative cell population were shown. (**B**) BM-isolated neutrophils isolated from Lysz-Cre mice and p38γ/$\delta^{Lyz\text{-}KO}$ transgenic mice were stimulated with FMLP 1μM or vehicle for 30 min. After stimulation, cells were stained with anti-L-selectin APC and anti-CD11b FITC. Signal was detected by flow cytometry. Histograms depict basal L-Selectin (left) or CD11b (right) membrane expression in Lysz-cre neutrophils (grey) and in p38γ/$\delta^{Lyz\text{-}KO}$ neutrophils (light blue) or fmlp induced L-Selectin (left) or CD11b (right) membrane expression in Lysz-Cre neutrophils (black) and in p38γ/$\delta^{Lyz\text{-}KO}$ neutrophils (dark blue). The shaded light grey line represents the isotype control. (**C**) Microscopy quantification of the rolling velocities of neutrophils recruited to slides covered with E-Selectin and ICAM-1. Data are means ± SEM. *P<0.05; ***P<0.001 (1-way ANOVA coupled to Bonferroni's post tests or *t-test*).



Appendix Fig. S8. Parabiosis assay, in which the circulation of a p38γ/δ$^{Lyzs\text{-}KO}$ mouse is shared with that of a WT partner mouse. CD45.1+ WT mice were sutured

to either CD45.2+ Lyzs-Cre or p38γ/δ$^{Lyzs\text{-}KO}$ mice by parabiosis surgery. Two weeks after surgery mice were fed a MCD diet for 3 weeks. (**A**) *Up*: Illustration of parabiotic couples. *Bottom*: Representative H&E stained liver sections from Lyzs-Cre (CD45.1), p38γ/δ$^{Lyzs\text{-}KO}$ (p38γ/δ$^{Lyzs\text{-}KO}$) and p38γ/δ$^{Lyzs\text{-}KO}$ (CD45.1) parabiotic mice. Scale bar: 50 μm. (**B**) Quantification of flow cytometry analysis of liver myeloid subsets (CD11b^{+} Gr-1^{high} (neutrophils) and CD11b^{+} Gr-1^{intermediate} (macrophages)) isolated from Lyzs-Cre (CD45.1), p38γ/δ$^{Lyzs\text{-}KO}$ (p38γ/δ$^{Lyzs\text{-}KO}$) and p38γ/δ$^{Lyzs\text{-}KO}$ (CD45.1) parabiotic mice. Bar charts show the diet-induced increase in each population relative to percentage of each subsets found in the blood of Lyzs-Cre (CD45.1), p38γ/δ$^{Lyzs\text{-}KO}$ (p38γ/δ$^{Lyzs\text{-}KO}$) and p38γ/δ$^{Lyzs\text{-}KO}$ (CD45.1) parabiotic mice. Data are means ± SEM. (n=5-10) **P<0.01 (1-way ANOVA coupled to Bonferroni's post tests).



Appendix Fig. S9. Neutrophil-specific p38δ deficiency protects against MCD-induced steatosis. (**A**) Immunoblot analysis of p38δ expression in bone marrow

neutrophils (10μg) and bone marrow-derived macrophages isolated (100 μg) from MRP8-Cre and p38δ$^{MRP8\text{-}KO}$ mice. MRP8-Cre and p38δ$^{MRP8\text{-}KO}$ mice were fed a MCD diet for 3 weeks. (**B**) Representative H&E and oil red stained liver sections. Scale Bar: 50μm. (n=5-10). (**C**) Plasma ALT at the end of the diet period.(**D**) Flow cytometry analysis of liver myeloid subsets (CD11b^{+} Gr-1^{high}, CD11b^{+} Gr-1^{intermediate}, CD11b^{+} Gr-1^{-}) isolated from MRP8-Cre and p38δ$^{MRP8\text{-}KO}$ mice after the diet. Representative dot plots are shown and bar charts of each population relative as a percentage of the total intra-hepatic CD11b^{+} leukocyte population. Data are means ± SEM (n=5-10). **P<0.01 (*t-test*).

Appendix Table S1. Characteristics of patients and controls

Variable	Obese patients with NAFLD (n = 73)	Controls without NAFLD (n = 11)	Controls with NAFLD (n= 9)
Age (years)	44.6 (10.2)	42.1 (10.8)	66.2 (12.8)**
Female:male ratio	52:21	7:4	2:7***
Hypertension (n)	36 (49.3)*	0	4 (44.4)
Diabetes mellitus (n)	22 (30.1)	0	1 (11.1)
BMI (kg/m^2)	49.1 (7.9)*	26.8 (5.0)	26.1 (2.8)
Fasting blood sugar (mg/dL)	108.7 (41.1)	89.8 (11.3)	101.8 (12.8)
AST (IU/L)	23.2 (11.7)	22.4 (4.7)	30.1 (18.,9)
ALT (IU/L)	30.4 (17.4)	25.3 (12.8)	46.3 (37.5)
Bilirubin (mg/dL)	0.4 (0.1)	0.6 (0.6)	0.7 (0.3)***
Albumin (mg/dL)	4.3 (0.3)	4.4 (0.3)	4.5 (0.6)***
Total cholesterol (mg/dL)	191.4 (36.6)	186.5 (36.1)	190.5 (50.4)
Triglycerides (mg/dL)	144.7 (80.6)	108.5 (51.9)	100.5 (32.5)
LDL-cholesterol (mg/dL)	112.2 (35.2)	105.1 (35.5)	125.7 (44.3)
HDL-cholesterol (mg/dL)	48.9 (16.1)	58.6 (19.0)	44.7 (13.1)
NAS score	5.1 (1.6)*	0	2.4 (2.1)**
Steatosis	2.2 (0.8)*	0	1.2 (1.2)**
Lobular inflammation	1.4 (0.9)*	0	0.6 (0.7)**
Hepatocyte ballooning	1.4 (0.6)*	0	0.8 (0.9)**

Variables are presented as mean (standard deviation) or absolute frequency (percentage) and are compared by means of Mann-Whitney U test or χ^2 test. NAFLD: non alcoholic fatty liver disease. BMI: body mass index. AST: aspartate aminotransferase. ALT: alanine aminotransferase. NAS: NAFLD Activity Score.

* Significantly different from other groups
**Significantly different from other groups
***Significantly different from obese with NAFLD group

APPENDIX MATERIALS & METHODS

Reagent

Anti-Ly6C/G antibody (BD Pharmigen), magnetic streptavidin microbeads (Miltenyi Biotec), DiD and DiO (Vybrant Cell-Labeling Solution, Molecular Probes), TNFα, aprotinin and leupeptin (Sigma), Ficoll (GE healthcare), haematoxylin and eosin (American Master Tech Scientific), protein-G-Sepharose (Amersham), OCT (Tissue-Tek), Red oil (American Master Tech Scientific), Fast sybr green (Applied Biosystems).

Parabiosis

CD45.1+ WT mice were sutured to either CD45.2+ Lyzs-Cre or p38γ/$\delta^{Lyzs\text{-}KO}$ mice and parabiosis surgery was performed as previously described (Casanova-Acebes et al, 2013). Briefly, mice were anesthetized with isofluorane, shaved at the corresponding lateral aspects and a longitudinal incision of skin was made along one side of each mouse. The olecranon and knee joints were attached by a single 5-0 polypropylene suture and tie, and the dorsal and ventral skins were approximated by continuous suture. After surgery, mice recieved a single dose of flunixin meglumine (1 mg/kg, Schering-Plough, Segré, France). Two weeks after parabiotic surgery, mice were fed a MCD diet for 3 weeks.

Immunoblot analysis

Tissue extracts were prepared in Triton lysis buffer [20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin and leupeptin]. Extracts (20-50 μg protein) and immunoprecipitates (prepared from 2-10 mg protein) were examined by immunoblot. For the immunoprecipitation assay,

liver extracts were incubated with 4 μg of anti-p38 delta or anti-p38 gamma coupled to protein-G-Sepharose. After incubation for 4 hours at 4°C, the captured proteins were centrifuged at 10,000 g, the supernatants discarded and the beads washed four times in lysis buffer. Beads were boiled for 5 minutes 95°C in 10μl sample buffer. The antibodies employed were anti-phospho p38 and anti-human p38δ (Cell Signaling); anti-p38γ and anti-p38δ (Gonzalez-Teran et al, 2013; Han et al, 2013); and anti-vinculin (Sigma). Immunecomplexes were detected by enhanced chemiluminescence (NEN).

Histology

Tissue samples were fixed in 10% formalin for 48h, dehydrated and embedded in paraffin. Sections (8μm) were cut and stained with haematoxylin and eosin (American Master Tech Scientific), picrosirius red (Sigma) and Masson trichromic (Merck). Tissue sections were sequentially stained with a biotinylated antibody to marker neutrophil activator nitrotyrosine (Hycultbiotech), streptavidin-conjugated horseradish peroxidase (Abcam), and the substrate 3,3'-diaminobenzidene (Vector Laboratories), followed by brief counterstaining with Mayer's haematoxylin (Sigma). Sections (8 μm) prepared from tissue frozen in OCT compound (Tissue-Tek) were stained with Red oil (American Master Tech Scientific).

Measurement of hepatic triglyceride

Hepatic triglyceride content was measured in livers from mice starved overnight. Total lipids were extracted from liver samples (25 mg) in an 8:1 mixture of chloroform and methanol (4 h). The extracts were mixed with 1N sulfuric acid and centrifuged (10 min). Triglycerides were measured with a commercial kit (Sigma).

Metabolic cages

Mice were housed under controlled temperature and lighting with free access to food and water. Food and water intake, energy expenditure, respiratory exchange ratio, and physical activity were monitored (3 days) in metabolic cages (TSE Systems, Bad Homburg, Germany). Fat and lean masses were detected by magnetic resonance imaging.

RNA-Seq Library Preparation, Sequencing, and Analysis

RNA-seq experiments were performed on biological quadruplicates. A total of 10 μg of total RNA was used for each RNA-seq library preparation according to the manufacturer's instructions (Illumina). RNA quality was verified using Bioanalyzer (Agilent); only RNA with a RIN of >9 was used. Libraries were prepared and sequenced (Illumina; GAII) in a pair-end, 36 bp format, except for the concentrated samples, which were sequenced by Hi-seq in a single-end, 50 bp format. Reads from each sample were aligned to the mouse genome (mm9 build) using TopHat (version 1.1.0). Differential expression was quantified using Cuffdiff (Trapnell et al., 2010) (version 1.0.3). Differentially expressed genes are those that have a log2 fold change of >0.58 or <0.58 and a q value of <0.05 compared with the control condition. We also required that the differentially expressed genes used for downstream analysis have a FPKM greater than 0.1 in the control condition. The raw data for the RNA-seq experiment were deposited in Gene Expression Omnibus, with the accession GSE58174.

RNA analysis

Expression of mRNA was examined by qRT-PCR using a 7900 Fast Real Time thermocycler and FAST SYBR GREEN assays (Applied Biosystems). Relative mRNA

expression was normalized to *Gapdh* mRNA measured in each sample. Primers used were as follows: *Mapk12* (Fw: AAGGGCTTTTACCGCCAGG; Rv: GGCGCAACTCTCTGTAGGC); *Mapk13* (Fw: ATGAGCCTCACTCGGAAAAGG; Rv: GCATGTGCTTCAAGAGCAGAA); *Lyzs* (Fw: ATGGAATGGCTGGCTACTATG; Rv: ACCAGTATCGG CTATTGATCT); *F4/80* (Fw: CCCCAGTGTCCTTACAGAGTG; Rv: GTGCCCAGA GTGGATGTCT); *Gr1* (Fw: GACTTCCTGCAACACAACTAC; Rv: ACAGCATTAC CAGTGATCTCA); *Tnfa* (Fw: CCCTCACACTCAGATCATCTT; Rv: GCTACGAC GTGGGCTACAG); *Il6* (Fw:TAGTCCTTCCTACCCCAATTT; Rv: TTGGTCCTTA GCCACTCCTTC); *Kc* (Fw: CTGGGATTCACCTCAAGAACA; Rv: CAGGGTCAA GGCAAGCCTC); *Ifng* (Fw: ATGAACGCTACACACTGCATC; Rv: CCATCCTTTTGCCAGTTCCTC); *Il10* (Fw: GCTCTTACTGACTGGCATGAG; Rv: CGCAGCTCTAGGAGCATGTG); *Il13* (Fw: CCTGGCTCTTGCTTGCCTT; Rv: GGTCTTGTGTGATGTTGCTCA); *Il23* (Fw: ATGCTGGATTGCAGAGCAGTA; Rv: ACGGGGCACATTATTTTTAGTCT); *Arg1* (Fw: CTCCAAGCCAAAGTCCTTAGAG; Rv: AGGAGCTGTCATTAGGGACATC); *Timp1* (Fw:GCAACTCGGACCTGGTCATAA; Rv: CGGCCCGTGATGAGAAACT); *Col1a1* (Fw: GCTCCTCTTAGGGGCCACT; Rv: CCACGTCTCACCATTGGGG); *Acta2* (Fw: GTCCCAGACATCAGGGAGTAA; Rv: TCGGATACTTCAGCGTCAGGA); *Csf1* (Fw: GGCTTGGCTTGGGATGATCCT; Rv: GAGGGTCTGGCAGGTACTC); *Cd68* (Fw: TGTCTGATCTTGCTAGGACCG; Rv: GAGAGTAACGGCCTTTTTGTG); *Ym1* (Fw: CAGGTCTGGCAATTCTTCTGA; Rv: GTCTTGCTCATGTGTGTAAGT); *Gapdh* (Fw: TGAAGCAGGCATCTGAGGG; Rv: CGAAGGTGGAAGAGTGGGA) (all mouse). Human primers used were as follows: *MAPK12* (Fw: CATGAGAAGCTAGGCGAGGAC; Rv: GCATCTGGTACACGAGGAACT);

MAPK13 (Fw: AAAAGGGCTTCTACAAGCAGG; Rv: TCGGGGACACGTAGGTCTT); *GAPDH* (Fw: CCATGAGAAGTATGACAACAGCC; Rv: GGGTGCTAAGCAGTTGGTG).

Neutrophils FACS analysis

Lyzs-Cre and p38γ/$\delta^{Lyzs\text{-}KO}$ neutrophils were isolated from bone marrow by labelling with biotin-conjugated anti-Ly6C/G antibody (BD Pharmigen) and magnetic streptavidin microbeads (Miltenyi Biotec), and then separating them on MACS columns (Miltenyi Biotec). Isolated Lyzs-Cre neutrophils were stimulated for 30 minutes with FMLP (1μM) or vehicle. Cells were stained with 1:100 anti–L-selectin APC and anti-CD11b FITC antibodies (BD Biosciences). For Lyzs-Cre and p38γ/$\delta^{Lyzs\text{-}KO}$ neutrophils isolation from the blood of parabiotic couples, mice were bled from cheeks, followed by red blood cells lysis and isolated neutrophils were stained with 1:100 anti-CD45.2 PerCP-Cy5.5 (eBioscience), anti-CD45.1 PE-Cy7, anti-Gr1 APC, anti-CD11b FITC and 1:5000 DAPI antibodies (BD Bioscience). MRP8-Cre and p38γ/$\delta^{MRP8\text{-}KO}$ neutrophils isolated from blood or liver were stained with anti-Gr1 APC and anti-CD11b FITC and 1:5000 DAPI antibodies. Signal was detected by flow cytometry.

Myeloid cells isolated from liver of Lyzs-Cre and p38γ/$\delta^{Lyzs\text{-}KO}$ mice 3 weeks after MCD diet were stained with anti-CD45.2 PerCP-Cy5.5, anti-Gr1 APC, anti-F4/80 PE-Cy7 and anti-CD11c FITC and biotin labeled anti-CD11b followed with streptavidin-eFluor450. Cells were sorted by using BDFACSAria II cytofluorimeter. Sorted liver myeloid subsets ($CD11b^+$ $Gr\text{-}1^{high}$, $CD11b^+$ $Gr\text{-}1^{intermediate}$, and $CD11b^+$ $Gr\text{-}1^-$) were stained with H&E and analyzed by using Leica DM2500 light microscope.

Blood and plasma analyses

Alanine transaminase (ALT) activity in plasma was measured using the ALT Reagent kit (Pointe Scientific). 3-Hydroxybutyrate levels were quantified using an enzymatic kit (Autokit 3-HB R1 and R2 sets, WAKO).

Blood glucose was measured with an Ascensia Breeze 2 glucose meter (Bayer), and percentages of circulating neutrophils and monocytes were measured with a Pentra80 hematologic analyser. Serum concentrations of cytokines were measured by multiplexed ELISA with a Luminex 200 analyser (Biorad).